Evaluation of chemicals for Toxic & Teratogenic effects using the chick embryo as the test system-FDA Contract #71-331 **(Calcium Saccharin)** FDA Compound #71-6
No Date

E26

F26

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

CALCIUM SACCHARIN: FDA 71-6

WARF INSTITUTE, INC.
Madison, Wisconsin

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no addjtions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasm gallispecticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transfered to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.

the hatcher was maintained at 98.5oF. and relative humidity at 86oF. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 88oF. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldahyde solution.

B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small

forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with ½" Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab", equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldahyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the 1" - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

C. Test Profile:

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

1. Preliminary Range Finding Experiments:

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range-Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

2. Dose-Response and Teratogenic Experiments:

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by X-ray. The chicks and embryos which had been X-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.

The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxilin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

Results:

The data developed in the testing of Calcium Saccharin are presented in the following tables:

Table 1 - Air Cell at 0 Hours

Table 2 - Air Cell at 96 Hours

Table 3 - Yolk at 0 Hours

Table 4 - Yolk at 96 Hours

Table 5 - Body Weight Data - Males

Table 6 - Body Weight Data - Females

Table 7 - Histopathology - Grow-Out Birds

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.

Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limbs or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these colums are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 3 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

Discussion:

The comments and data which follow concern the results obtained when Calcium Saccharin was employed in the test system.

Significant toxicity (P.05) was seen in the 0 hour air cell treatments at 110.0, 183.3, 366.7, 440.0 and 733.3 mg/kg when compared with the solvent treated controls. The calculated LD-50 was 667.0 mg/kg. At 96 hour air cell the toxicity was significantly elevated at 24.4, 36.7, 73.3, 110.0, 122.2, 146.7, 220.0 and 440.0 mg/kg with an LD-50 of 31.9 mg/kg.

In 0 hour yolk treatments, a single instance of significantly increased mortality (toxicity) was seen at 183.3 mg/kg with a calculated LD-50 of 267,884 mg/kg. The highest dose administered at this time and route was 733.3 mg/kg. In the 96 hour yolk treatments the mortality was not significantly increased as a result of treatment when compared with the solvent controls.

Significantly increased levels of abnormalities were seen at all treatment times and routes with the exception of 96 hour air cell.

In the 0 hour air cell treatments, growth retardation was a principal contributor to the total abnormalities with from 8 to 32 percent of the embryos involved in a particular dose level. More striking was the pale down color seen in the chicks which hatched. The degree of hypopigmentation was such that the down color of hatched chicks was "chalk white" at the highest dose level, 733.3 mg/kg and progressively less severe at 366.7, 183.3 and 110.0 mg/kg. The severity of the effect was generally dose related. The hypopigmentation was not seen below 110.0 mg/kg. A scattering of other abnormalities such as parrot beak, crossed beak and celosomia were seen at 733.3 mg/kg, but were low in occurrence.

In 96 hour air cell treatments mortality was very high and in the few chicks which hatched at 73.3 through 220.0 mg/kg, the same hypopigmentation was seen as in the 0 hour air cell chicks. Several instances of growth retardation were observed. Flexed maxilla, parrot beak, short beak were seen at 9.8 mg/kg, a single occurrence of microblepharia and exencephaly were noted.

At 0 hour yolk, hypopigmentation was the major contributor to total abnormalities with the majority of the chicks involved at the upper 5 dose levels. The severity of this abnormality was generally dose related. The instances of retarded development were low in number and essentially equal to the level seen in the flock background.

In the 96 hour yolk treatments, the hypopigmentation seen was dose related and affected the chicks which hatched at the upper 5 dose levels. The level of retarded development observed was essentially equal to the flock background.

In both the 0 and 96 hour yolk treatments a very low incidence of other abnormalities was seen and included parrot beak, crooked sternum, curled toes, edema, clubbed down, bulla, exencephaly and adsence of one kidney. A single occurrence of each abnormality was noted.

Dwarfism, growth retardation, was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6, we did not classify any embryos as retarded unless they were alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism. One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. In this experiment the chicks which hatched were of normal size and development.

The normal down color for chicks of this breed is pale yellow. In general, the hypopigmentation observed in hatched chicks was most severe at the upper dose levels where the chicks were "chalk white." The degree of paleness was dose related so that as the dose level decreased an improvement in down color was seen.

The post hatch chicks were raised to 10 weeks of age and included birds from the untreated control and various treatment groups as indicated in the attached tables. Body weight gains and feed consumption were considered normal in all groups. As the chicks with pale down became older and their final feathering was established, the feather color was normal.

Histopathological examination was conducted on tissues from birds in the 0-hour yolk - 366.7 mg/kg and 96 hour air cell - 73.3 mg/kg. The tissues were compared with those of similar age untreated controls. The alterations seen were minimal in nature and rather randomly distributed among the various groups.

X-ray examinations did not reveal abnormalities not already noted on gross examinations.

Conclusion:

Under the conditions specified for this trial, Calcium Saccharin was particularly toxic at the 0 hour air cell administration and produced significantly elevated levels of abnormalities in the 0 hour air cell and both the 0 and 96 hour yolk treatments.

Signed RM Bodden

By and For WARF Institute, Inc.

October 1, 1974

Test Sample: Calcium Saccharin

Identification: FDA 71-6

Solvent System: 50% Absolute Ethanol in sterile distilled H_2O

Breeder Flock: N-1

Preliminary Range Finding Experiments

Experiment No.	Initiated
8	12-16-71
21	3-30-72

Dose Response Experiments

Experiment No.	Initiated
28	5-15-72
43	8-21-72

Table 1
Calcium Saccharin
Air Cell At 0 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
733.3	50	92.00*	50.00*	12.00
440.0	18	55.55*	77.77*	.00
366.7	109	28.44*	79.81*	.91
220.0	30	23.33	76.66*	3.33
183.3	99	27.27*	79.79*	6.06
110.0	130	18.46*	68.46*	.00
73.3	20	25.00	10.00	5.00
36.7	123	13.82	10.56	1.62
14.7	60	20.00	13.33*	6.66
50% Ethanol	209	10.04	4.78	3.34
Drilled Control	80	20.00	12.50	6.25
Control/ Control	280	9.28	4.28	1.78

** LD-50 667.0 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 2
Calcium Saccharin
Air Cell At 96 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
440.0	19	100.00*	.00	.00
220.0	20	95.00*	.00	.00
146.7	99	97.97*	7.07	3.03
122.2	10	100.00*	.00	.00
110.0	19	89.47*	.00	.00
73.3	129	81.39*	4.65	.00
36.7	30	43.33*	3.33	.00
24.4	100	61.00*	8.00	.00
12.2	10	.00	.00	.00
9.8	100	22.00	10.00	2.00
4.9	110	11.81	10.00	2.72
50% Ethanol	209	18.18	10.52	2.39
Drilled Control	80	7.50	7.50	1.25
Control/ Control	280	9.28	4.28	1.78

** LD-50 31.9 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 3
Calcium Saccharin
Yolk At 0 Hour

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
733.3	50	38.00	74.00*	.00
366.7	110	29.09	70.00*	.90
220.0	10	20.00	80.00*	.00
183.3	99	38.38*	65.65*	5.05
110.0	110	30.00	71.81*	.90
36.7	109	29.35	6.42	3.66
14.7	59	20.33	6.77	3.38
50% Ethanol	150	22.66	8.00	4.00
Pierced Control	60	35.00	15.00	3.33
Control/ Control	280	9.28	4.28	1.78

** LD-50 267,884 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 4
Calcium Saccharin
Yolk At 96 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
733.3	50	24.00	84.00*	4.00
366.7	110	14.54	87.27*	4.54
220.0	10	.00	90.00*	.00
183.3	99	22.22	93.93*	3.03
110.0	110	16.36	86.36*	4.54
36.7	110	12.72	20.00	4.54
14.7	60	10.00	3.33	1.66
50% Ethanol	149	21.47	12.75	3.35
Pierced Control	60	18.33	13.33	5.00
Control/ Control	280	9.28	4.28	1.78

** LD-50 not calculable > 733.3 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 5 Body Weight Data - Post Hatch Response

FDA 71-6: Calcium Saccharin

(Males)

Test (1) Dose	Time/Route	Average Individual Body Weight - Grams						
		Week 1	2	3	4	6	8	10
---	Control/Control	75	148	222	322	586	839	1142
366.7	0/AC	74	141	230	330	536	834	1084
36.7	0/AC	78	145	222	322	551	835	1091
366.7	0/Y	73	140	204	302	556	800	1121
36.7	0/Y	81	152	228	340	597	874	1163
146.7	96/AC	73	140	217	327	578	861	1089
4.9	96/AC	75	147	226	328	594	859	1140
366.7	96/Y	79	148	218	318	547	788	1117
14.7	96/Y	68	146	224	323	590	900	1216

(1) Milligrams/Kilograms of body weight

Table 6 Body Weight Data - Post Hatch Response

FDA 71-6: Calcium Saccharin

(Females)

Test (1) Dose	Time/Route	Average Individual Body Weight - Grams						
		Week 1	2	3	4	6	8	10
---	Control/Control	75	139	206	300	493	714	934
366.7	0/AC	68	125	186	272	475	648	846
36.7	0/AC	72	132	198	298	494	716	903
366.7	0/Y	73	136	218	295	505	700	883
36.7	0/Y	79	143	211	316	528	739	938
146.7	96/AC	76	140	213	315	532	751	950
4.9	96/AC	77	143	217	315	534	749	949
366.7	96/Y	78	138	218	299	499	669	902
14.7	96/Y	71	131	205	281	496	685	878

(1) Milligrams/Killograms of body weight

Table 7
Calcium Saccharin
Histopathology - Grow-Out Birds

Histologic Observation	Negative Control (10)	O/Y 366.7 MG/KG (10)	96/A 73. MG/KG (1
Thyroid			
cellular infiltration/ increased cellularity	2	1	2
congestion			1
less colloid		2	1
Lungs			
congested	3		
Heart			
cellular infiltration	1	1	
Spleen			
pigmentation	8	8	9
Liver			
pigmentation	7	1	3
cellular infiltration	9	3	3
degenerate		1	
Pancreas			
degenerate	1	1	
Kidney			
cellular infiltration		1	
atrophic glomeruli			1
Testicles			
immature	1	1	4
degenerate	1	2	
irregular tubules		1	
Ovaries			
shrunken follicles		1	
degenerate		1	
immature		2	

Table 7 (Continued)
Calcium Saccharin
Histopathology - Grow-Out Birds

Histologic Observation	Negative Control (10)	O/Y 366.7 MG/KG (10)	96/A 73. MG/KG (1
Bone Marrow			
numerous fat vacuoles	2		